Transcriptional Induction of Cyclooxygenase-2 in Osteoblasts Is Involved in Interleukin-6-Induced Osteoclast Formation* 

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ABSTRACT

Interleukin-6 (IL-6) induces osteoclast-like cell (osteoclast) formation in a dose-dependent fashion in cocultures of mouse bone marrow cells and osteoblastic cells when soluble IL-6 receptor (sIL-6R) is present. Simultaneous treatment with submaximal doses of IL-1α and IL-6 with sIL-6R caused marked induction of osteoclast formation and PGE₂ synthesis. These effects were suppressed by adding neutralizing antibodies against IL-1α or IL-6R and were totally abolished by adding nonsteroidal antiinflammatory drugs, such as indomethacin and a selective cyclooxygenase-2 (COX-2) inhibitor (NS398). In mouse osteoblastic cells, both IL-1α and IL-6 with sIL-6R markedly induced messenger RNA expression of COX-2, but not COX-1, as determined by Northern blot analysis, and luciferase activity in cells stably transfected with a COX-2 promoter-luciferase fusion construct. IL-6 and sIL-6R, when added separately, did not stimulate COX-2 messenger RNA expression. Simultaneous addition of IL-1α and IL-6 with sIL-6R to osteoblast cultures cooperatively induced transcription of COX-2, which was associated with a marked increase in COX activity measured by the conversion of arachidonic acid into PGE₂. The increased PGE₂ synthesis by osteoblasts may play an important role in osteoclastogenesis induced by submaximal doses of IL-1 and IL-6. (Endocrinology 138: 2372–2379, 1997)

Bone resorption is stimulated by several local factors including interleukin-1 (IL-1), IL-6, IL-11, leukemia inhibitory factor, tumor necrosis factor-α (TNFα), and PGE₂ (1–3). IL-1 markedly stimulates osteoclastic bone resorption in vitro and in vivo by enhancing both osteoclast formation and function (1–4). It is known that PGE₂ production by osteoblasts is involved in the mechanism of osteoclastic differentiation induced by IL-1 (3, 4). Other cytokines, including IL-6, IL-11, and leukemia inhibitory factor, all of which transduce their signals through the signal-transducing gp130 chain, also induce osteoclast formation in vitro (5–7). IL-6 appears to act on osteoblast cells, but not osteoclast progenitors, to induce osteoclast differentiation (7). We reported that the presence of soluble IL-6 receptor (sIL-6R) was essential for osteoclast formation by IL-6 in cocultures of mouse bone marrow cells and osteoblastic cells (5, 7) due to the lack of membrane-bound IL-6 receptors in osteoblasts under physiological conditions. In the course of examining the mechanism of osteoclast differentiation by IL-6 and IL-1, we found that submaximal doses of the two cytokines, neither of which alone had much of an effect at that dosage, greatly stimulated osteoclast formation in cocultures of bone marrow cells and osteoblastic cells. This suggests that there is an interaction between IL-1 and IL-6 in inducing osteoclast differentiation. However, no common mechanism of IL-1 and IL-6 action in inducing osteoclast differentiation has been reported.

PG synthesis is regulated by two successive metabolic steps; the release of arachidonic acid from membranous phospholipids and its conversion to prostanooids. Phospholipase A2 is the enzyme responsible for arachidonic acid release, and cyclooxygenase (COX) is a rate-limiting enzyme for the conversion of arachidonic acid to prostanooids (8, 9). Two COX genes, COX-1 and COX-2, have been identified (10, 11). COX-1 is constitutively expressed in many mammalian tissues (10). In contrast, COX-2 is generally undetectable under physiological conditions, but is markedly induced by several cytokines and growth factors (8–11). Both COX-1 and COX-2 are expressed in osteoblastic cells. We reported that IL-1 stimulates messenger RNA (mRNA) expression of COX-2, but not COX-1, in osteoblastic cells, and that COX-2 is the major enzyme regulating PG synthesis in response to several bone-resorbing factors, such as IL-1, basic fibroblast growth factor, and PGE₂ (12–15). Therefore, COX-2-dependent PG synthesis by osteoblasts is considered to play a key role in bone resorption associated with inflammation.

Estrogen deficiency causes a marked bone loss by stimulating osteoclastic bone resorption. Recent studies have focused on the involvement of IL-1 and IL-6 in osteoclastic
bone resorption due to estrogen deficiency. The administration of IL-1 receptor antagonist to ovariectomized (OVX) rats decreased bone loss (16, 17). The increased number of osteoclasts in OVX mice was normalized by administration of a neutralizing antibody against IL-6 (18, 19). We reported that the bone-resorbing activity present in bone marrow supernatants from OVX mice was much higher than that in bone marrow supernatants from sham mice (20). In mouse calvarial cultures, bone marrow supernatants from OVX mice had a greater effect on COX-2 mRNA expression and PGE$_2$ synthesis than those from sham mice (21). These results suggest that PGE$_2$ is also involved in bone resorption due to estrogen deficiency.

In this study, we examined the possible involvement of PGE$_2$ synthesis by osteoblastic cells in IL-6-induced osteoclast formation. Both IL-1 and IL-6 in the presence of sIL-6R cooperatively stimulated osteoclast formation and PGE$_2$ production in cocultures of mouse bone marrow cells and osteoblastic cells. In osteoblasts, not only IL-1, but also IL-6 in the presence of sIL-6R, markedly stimulated COX-2 gene transcription. IL-1 and IL-6 with sIL-6R cooperatively induced COX-2 expression, which resulted in marked stimulation of COX activity. The COX-2-dependent PG synthesis involved in osteoclastogenesis may be additively induced by IL-1 and IL-6.

**Materials and Methods**

**Animals and drugs**

Newborn and 7-week-old male mice of the ddy strain were obtained from Shizuoka Laboratory Animal Center (Shizuoka, Japan). Mice were used according to the regulations approved by the institutional animal care committee. Recombinant human IL-1α and neutralizing antibody against human IL-1α were purchased from Genzyme (Cambridge, MA). Recombinant mouse IL-6 and recombinant mouse sIL-6R were prepared from Chinese hamster ovary cells transfected with a mouse IL-6 complementary DNA (cDNA) expression vector and a mouse sIL-6R cDNA expression vector, respectively, as previously reported (S). Recombinant human IL-6 and human sIL-6R were purchased from R&D Systems (Minneapolis, MN). Neutralizing antibody against mouse IL-6R (MR16–1) was prepared as described previously (22). Arachidonic acid was purchased from Sigma Chemical Co. (St. Louis, MO). NS398 was purchased from Calbiochem (La Jolla, CA). All other chemicals were of analytical grade.

**Culture of primary mouse osteoblastic cells**

Primary osteoblastic cells were isolated from 1-day-old mouse calvaria after five routine sequential digestions with 0.1% collagenase (Genzyme, Tokyo, Japan) as previously described (12). Osteoblasts isolated from the fractions 3–5 were combined and cultured in αMEM supplemented with 10% FBS at 37°C in a humidified atmosphere of 5% CO$_2$ in air.

**Coculture of mouse bone marrow cells and osteoblastic cells**

Primary osteoblastic cells (1 × 10$^5$) were cocultured with bone marrow cells (2 × 10$^5$) for 7 days in the well of 48-well culture plates with 0.3 ml αMEM containing 10% FBS as previously reported (3, 5). On day 4, medium was changed to the respective fresh medium containing each test chemical. On day 7, adherent cells were fixed and stained for tartrate-resistant acid phosphatase (TRAP), and the number of TRAP-positive osteoclast-like multinucleated cells formed was counted as previously described (3, 5). The conditioned media collected on days 4 and 7 were combined and used for the determination of PGE$_2$ levels.

**Measurement of PGE$_2$ content**

The concentration of PGE$_2$ in the culture medium was determined using a RIA kit (NEK-020, DuPont-New England Nuclear, Boston, MA). The detection limit of the PGE$_2$ RIA kit was 0.5 ng/ml.

**Northern blot analysis**

Primary osteoblastic cells were cultured for 24 h in αMEM supplemented with 0.1% FBS, then incubated for 3 h with or without cytokines. A DNA construct containing 371 bp of the murine COX-2 promoter and 70 bp of downstream untranslated DNA fused to a luciferase reporter gene (23) was kindly provided by Dr. H. Herschman (University of California, Los Angeles, CA). A mouse osteoblastic cell line, MC3T3-E1, stably transfected with this construct, as described below, was cultured for 24 h in serum-free DMEM, then incubated for 30–180 min with or without cytokines. Total cellular RNA was extracted using the acid guanidium-phenol-chloroform method (12). For Northern blotting, 10 μg total RNA were resolved by electrophoresis in a 1% agarose-formaldehyde gel and transferred onto nylon membranes (Hybond N, Amersham, Arlington Heights, IL), then hybridized with a 32P-labeled cDNA probe as previously reported (12). The signals were densitometrically quantified using a Bioimage analyzer (BAS-2000, Fuji Film, Tokyo, Japan). Mouse COX-1 and COX-2 cDNA probes were purchased from Oxford Biomedical Research (Oxford, MI). Mouse COX-2 cDNA was provided by Dr. H. Herschman (University of California, Los Angeles), and mouse COX-1 cDNA was provided by Dr. W. Smith (Michigan State University, East Lansing, MI). Mouse IL-6 and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes were amplified by PCR using the respective amplier sets from Clontech (Palo Alto, CA). Luciferase cDNA was amplified by PCR (sense primer, 5'-CGCTTGAAGCAGAAGCATAGGTATG-3'; antisense primer, 5'-TAGTCTCAGTGAGCCCATATCCTTG-3').

**Assay for COX activity**

Primary osteoblastic cells and MC3T3-E1 cells were cultured for 15 h on 48-well culture plates containing αMEM supplemented with 0.1% FBS, then treated for 5 h with or without cytokines in αMEM containing 0.1% FBS. The end of culture, cells were washed twice with αMEM and incubated for 15 min with 10 μM arachidonic acid in αMEM containing 0.1% FBS. Conditioned media were collected for the determination of PGE$_2$ levels.

**Determination of COX-2 promoter activity**

The COX-2 promoter-luciferase fusion construct containing 371 bp of the 5′-flanking sequence and 70 bp of downstream untranslated DNA (P2-Luc371) (23) was purified by CsCl banding and cotransfected with pSV2-neo into cultured MC3T3-E1 cells using Lipofectamine (Life Technologies, Grand Island, NY). After selection using G418 for 2 weeks, stable colonies were pooled and used for luciferase assay as previously reported (13). Cells (5 × 10$^5$) were plated in six-well dishes and grown for 6 days in DMEM containing 10% FBS. They were precultured for 24 h in serum-free DMEM with 1 mg/ml BSA, then treated with human IL-1α and/or human IL-6 with sIL-6R. Luciferase activity was measured in soluble cell extracts prepared with a luciferase detection kit (Promega, Madison, WI) using an automatic injection luminometer (Berthold Lu-mat, Wallac, Gaithersburg, MD). Activity was normalized to total protein measured with a BCA protein assay kit (Pierce Chemical Co., Rockford, IL).

**Statistical analysis**

Statistical analysis was carried out using ANOVA, and the significance of differences between two groups was determined by a post-hoc test using the Bonferroni/Dunn method.

**Results**

**Cooperative effects of IL-1 and IL-6 on osteoclast formation**

IL-1 and IL-6 have been implicated as factors influencing bone resorption in several metabolic bone diseases, such as...
postmenopausal osteoporosis and rheumatoid arthritis (17, 19, 20, 24, 25). To examine further the effects of IL-1 and IL-6 on osteoclastogenesis, we used a coculture of mouse bone marrow cells and osteoblastic cells. We reported that IL-6 markedly stimulates osteoclast formation in the presence of sIL-6R in the coculture (5). IL-1α, at 30–1000 pg/ml, also induces osteoclast formation in this coculture system (3). When submaximal doses of IL-1α (30 pg/ml) or IL-6 (20 ng/ml) together with sIL-6R (250 ng/ml) were separately added to the coculture, osteoclast formation was detected, but only slightly (Fig. 1). Simultaneous addition of these cytokines caused a marked cooperative effect on osteoclast formation, and the potency was equivalent to the maximal effects induced by either 1000 pg/ml IL-1α or 200 ng/ml IL-6 with sIL-6R (250 ng/ml; Fig. 1). Furthermore, treatment with a submaximal dose (30 pg/ml) of IL-1α and a maximal dose (200 ng/ml) of IL-6 with sIL-6R (250 ng/ml) had an even greater effect on osteoclast formation (Fig. 1).

Figure 2 shows the effects of neutralizing antibodies against human IL-1α and mouse IL-6R on osteoclast formation induced by submaximal doses of IL-1α (30 pg/ml) and IL-6 (20 ng/ml) in the presence of sIL-6R (250 ng/ml). These neutralizing antibodies have been proven to abolish specifically the biological function of IL-1α and IL-6, respectively (22). Both antibodies suppressed the osteoclast formation induced by submaximal doses of IL-1α and IL-6 in the presence of sIL-6R when they were added separately (Fig. 2).

PGE₂ production is essential for osteoclast formation cooperatively induced by IL-1 and IL-6

We previously reported that PGE₂ produced by osteoblasts is involved in IL-1-induced osteoclast formation in coculture of bone marrow cells and osteoblastic cells (3). However, it was not known whether PGE₂ production was involved in IL-6-induced osteoclast formation. Figure 3 shows the effects on PGE₂ production of IL-1α and IL-6 with sIL-6R added separately or in combination to the coculture. PGE₂ production was examined by measuring the level of PGE₂ in conditioned medium, as shown in Fig. 1. Not only IL-1α, but also IL-6, in the presence of sIL-6R markedly stimulated PGE₂ production in the cocultures (Fig. 3). When submaximal doses of IL-1α (30 pg/ml) and IL-6 (20 ng/ml) together with sIL-6R (250 ng/ml) were added simultaneously, PGE₂ production was increased to a level equivalent to that induced by 1000 pg/ml IL-1α (Fig. 3).

To examine further the involvement of PGE₂ production in osteoclast formation cooperatively induced by IL-1 and IL-6, we tested the effects of nonsteroidal antiinflammatory drugs (indomethacin and NS-398) on osteoclast formation. Indomethacin is an inhibitor of both COX-1 and COX-2, and NS-398 is a selective inhibitor of COX-2 (26). Both indomethacin and NS-398 completely suppressed osteoclast formation induced by submaximal doses of IL-1α (30 pg/ml) and IL-6 (20 ng/ml) in the presence of sIL-6R (250 ng/ml; Fig. 4). Under these conditions, the level of PGE₂ produced by low doses of IL-1 and IL-6 was 35 ng/ml, and this was completely
Expression of COX-2 mRNA in mouse osteoblastic cells

COX-1 and COX-2 are rate-limiting enzymes for PG synthesis, and IL-1 greatly induces COX-2 expression in osteoblastic cells (12, 15). To examine the effects of IL-6 on mRNA expression of COX-1 and COX-2 in osteoblasts, primary mouse osteoblasts were cultured in αMEM containing 0.1% FBS for 24 h, then treated for 3 h with or without IL-6 and sIL-6R. IL-6 (200 ng/ml) in the presence of sIL-6R (250 ng/ml) significantly induced COX-2 mRNA expression (Fig. 5). When submarginal doses of IL-1α (30 pg/ml) and IL-6 (20 ng/ml) with sIL-6R (250 ng/ml) were separately added to the coculture, there was slight expression of COX-2 mRNA. sIL-6R (250 ng/ml) alone had no effect. Simultaneous addition of the submarginal doses of IL-1α and IL-6 together with sIL-6R caused a marked increase in COX-2 mRNA expression in osteoblasts. The potency was greater than that induced by 1000 pg/ml IL-1α (Fig. 5).

To confirm the cooperative effects of IL-1 and IL-6 on COX activity, we measured the conversion of arachidonic acid into PGE2 in osteoblasts. After primary osteoblastic cells were cultured for 5 h with or without IL-1α and/or IL-6 in the presence of sIL-6R in αMEM containing 0.1% FBS, they were washed and incubated for 15 min in αMEM containing 10 μM arachidonic acid. The COX activity, as measured by PGE2 production, was induced not only by IL-1α but also by IL-6 in the presence of sIL-6R (Table 1). Consistent with the data shown in Fig. 5, simultaneous treatment with submarginal doses of IL-1α (30 pg/ml) and IL-6 (20 ng/ml) in the presence of sIL-6R (250 ng/ml) induced even greater COX activity than that produced by a high dose of IL-1 (Table 1). This indicates that the COX-2 enzyme induced by submarginal doses of IL-1 and IL-6 together with sIL-6R is capable of converting arachidonic acid into PGE2 in osteoblastic cells.

Transcriptional regulation of the COX-2 promoter by IL-6

To examine transcriptional regulation of the COX-2 promoter by IL-6, MC3T3-E1 cells were stably transfected with a COX-2 promoter-luciferase fusion construct containing 371 bp of 5′-flanking sequence (P2-Luc371). IL-6 (200 ng/ml) stimulated luciferase activity in transfected cells at 3 h in the presence of sIL-6R, the potency of which was similar to that suppressed by adding indomethacin (data not shown). When the same amount (35 ng/ml) of PGE2 was added to the coculture with indomethacin or NS-398, osteoclast formation was completely restored (Fig. 4). These results indicate that the endogenous production of PGE2 is a prerequisite for the osteoclast formation induced by submaximal doses of IL-1α and IL-6 with sIL-6R.
induced by IL-1α (10 ng/ml; T/C ratios for luciferase activity were 2.8 and 3.1, respectively). Neither IL-6 nor sIL-6R stimulated luciferase activity, when they were added separately. Simultaneous treatment with IL-1α (10 ng/ml) and IL-6 (200 ng/ml) in the presence of sIL-6R (250 ng/ml) enhanced luciferase activity in the cells transfected with P2-Luc371 (T/C ratio was 6.2; Fig. 6).

Finally, a time-course study of Northern blot analysis for luciferase, COX-1, and COX-2 mRNAs was performed using MC3T3-E1 cells transfected with P2-Luc371. Expression of both luciferase and COX-2 mRNAs was stimulated by IL-6 in the presence of sIL-6R at 30 min, and the levels were higher than the control value until 180 min (Fig. 7). IL-1α also stimulated the expression of luciferase and COX-2 mRNAs at 30–180 min. Simultaneous treatment with IL-1α and IL-6 in the presence of sIL-6R cooperatively induced the expression of luciferase and COX-2 mRNAs in MC3T3-E1 cells transfected with P2-Luc371 (Fig. 7). Expression of COX-1 mRNA could be detected in the cells, but it was not affected appreciably by treatment with IL-1 and/or IL-6 (Fig. 7). In addition, we found that IL-6 with sIL-6R stimulated IL-6 mRNA expression at 30–60 min (Fig. 7). As reported previously, IL-1α induced IL-6 mRNA expression at 120–180 min. The expression of IL-6 mRNA was synergistically enhanced by simultaneous addition of IL-1α and IL-6 in the presence of sIL-6R at 30–60 min, suggesting that autoamplification of the IL-6 gene by IL-6 is involved in the mechanism of additive production of PGE₂ by IL-1 and IL-6.

**Discussion**

The present study clearly indicates that IL-6 induces COX-2 gene transcription and PGE₂ synthesis in osteoblasts in the presence of sIL-6R. Osteoclast formation induced by IL-6 and sIL-6R was accompanied with PGE₂ production in coculture of bone marrow cells and osteoblastic cells, which was blocked completely by a selective COX-2 inhibitor (NS-398). Simultaneous treatment with submaximal doses of IL-1α and IL-6 in the presence of sIL-6R cooperatively induced osteoclast formation in the coculture as well as PGE₂ production by osteoblasts. Osteoclast formation induced by IL-1 and/or IL-6 appeared to depend on their ability to induce COX-2 gene transcription in osteoblasts.

We reported that IL-6 alone could not induce osteoclast formation in cocultures of mouse bone marrow cells and...
TABLE 1. Effects of IL-1, IL-6, and sIL-6R on COX activity of mouse primary osteoblastic cells and MC3T3-E1 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Primary osteoblastic cells</th>
<th>T/C ratio</th>
<th>MC3T3-E1 cells</th>
<th>T/C ratio</th>
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<tbody>
<tr>
<td>Control culture</td>
<td>1.00 ± 0.16</td>
<td></td>
<td>1.00 ± 0.13</td>
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<tr>
<td>IL-1 30 pg/ml</td>
<td>2.54 ± 0.72&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.30 ± 0.22</td>
<td>3.35 ± 0.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>1000 pg/ml</td>
<td>4.17 ± 0.51&lt;sup&gt;a,b&lt;/sup&gt;</td>
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<tr>
<td>IL-6 20 ng/ml</td>
<td>1.08 ± 0.45</td>
<td>0.96 ± 0.16</td>
<td></td>
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</tr>
<tr>
<td>200 ng/ml</td>
<td>1.80 ± 0.45</td>
<td>0.95 ± 0.09</td>
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<tr>
<td>sIL-6R 250 ng/ml</td>
<td>1.21 ± 0.37</td>
<td>0.89 ± 0.21</td>
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<tr>
<td>sIL-6R 250 ng/ml +</td>
<td>2.56 ± 0.83&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.21 ± 0.28</td>
<td></td>
<td></td>
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<tr>
<td>IL-6 200 ng/ml</td>
<td>3.37 ± 1.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.55 ± 0.19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1 30 pg/ml + sIL-6R 250 ng/ml</td>
<td>5.81 ± 0.88&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>1.95 ± 0.38&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>2.38 ± 0.41&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>IL-6 20 ng/ml</td>
<td>8.76 ± 2.30&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>sIL-6R 200 ng/ml</td>
<td>9.87 ± 2.09&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
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Primary osteoblastic cells or MC3T3-E1 cells were cultured for 5 h with or without IL-1, IL-6, and sIL-6R, separately or in combination, in αMEM containing 0.1% FBS, then incubated for 15 min with 10 μM arachidonic acid. The levels of PGE2 in the control culture from primary osteoblastic cells and MC3T3-E1 cells were 0.5–2 and 10–15 ng/ml, respectively. The level of PGE2 is expressed as the ratio of the culture treated with cytokines to the control culture (T/C ratio). Data are shown as the mean ± SD of three experiments with four to six cultures.

<sup>a</sup> P < 0.01 vs. control.
<sup>b</sup> P < 0.05 vs. culture treated with 30 pg/ml IL-1.
<sup>c</sup> P < 0.05 vs. culture treated with IL-6 and sIL-6R.

![Graph showing time course of change in the expression of luciferase, COX-2, IL-6, and GAPDH](image)

Fig. 7. Time course of change in the expression of luciferase, COX-2, COX-2, and IL-6 mRNAs induced by IL-1α, IL-6, and sIL-6R in MC3T3-E1 cells stably transfected with the COX-2 promoter-luciferase fusion construct. MC3T3-E1 cells stably transfected with a 371-bp COX-2 promoter-luciferase fusion construct were serum deprived for 24 h and treated for 30–180 min with or without IL-1α and IL-6 in the presence of sIL-6R, separately or in combination. Total RNA was extracted and Northern blotted using 32P-labeled cDNA probes of luciferase, COX-1, COX-2, IL-6, and GAPDH.

osteoblastic cells, but sIL-6R strikingly triggered osteoclast formation by IL-6 (5). We also reported that mouse primary osteoblastic cells expressed a very low level of membrane-bound IL-6 receptors, which was not enough for IL-6-mediated signal transduction (7). In this study, IL-6 alone stimulated neither COX-2 mRNA expression nor COX-2 promoter activity in osteoblastic cells, but sIL-6R triggered COX-2 mRNA expression by IL-6. This is consistent with the previous findings on osteoclast formation and signal transduction of gp130-related cytokines in osteoblasts (27). Romas et al. (28) reported that IL-1, TNFα, PGE₂, PTH, and 1α,25-dihydroxyvitamin D₃ similarly induced IL-11 production by osteoblasts, and that neutralizing antibody against mouse gp130 inhibited osteoclast formation induced by these factors completely or partially. Girasole et al. (6) also reported that PTH and 1α,25-dihydroxyvitamin D₃ stimulated IL-11 production in bone marrow stromal cell cultures, and antibody against IL-11 suppressed osteoclast formation induced by these factors. In the present study, osteoclast formation induced by submaximal doses of IL-1 and IL-6 in the presence of sIL-6R was completely suppressed by indomethacin and a selective COX-2 inhibitor (NS-398). Osteoclast formation induced by IL-11 was also inhibited by indomethacin (data not shown), indicating that PGE₂ production is essential for IL-11-induced osteoclast formation. Pilbeam et al. (14) reported autoamplification of COX-2 in osteoblastic cells by PGE₂. This suggests that PGE₂ produced by osteoblasts in response to IL-1 and IL-6 stimulates COX-2 expression in osteoblasts, which, in turn, causes a marked increase in PGE₂ synthesis.

PGE₂ production by osteoblasts can be regulated by many factors, including IL-1, PTH, basic fibroblast growth factor, TGFs, and PGE per se (12–15, 29, 30). COX-2 expression appears responsible for bone resorption induced by some of these factors (12, 13). The present study suggests that COX-2 is preferentially responsible for the IL-1-induced PG synthesis by osteoblasts. However, the possibility that COX-1 is also involved in the IL-1-induced PG synthesis by osteoblasts cannot be ruled out completely at present, as constant expression of COX-1 mRNA was detected as well. The COX-1 protein was weakly, but constantly, expressed in osteoblasts, but it was not affected by treatment with IL-1 (31).

We found that IL-6 induces transcriptional activation of COX-2 in the presence of sIL-6R in osteoblasts. The 5′-flanking region of the COX-2 gene promoter contains various putative transcriptional regulatory elements such as cAMP responsive element, nuclear factor IL-6 (NF-IL-6), activator protein-2, specificity protein 1 (Sp1), and nuclear factor-kB (NFκB) (32, 33). Of these regulatory elements, cAMP response element, and NF-IL-6 have been reported to act as positive regulatory elements for COX-2 transcription (30, 32).
In mouse osteoblasts, Yamamoto et al. (34) reported that both NF-IL-6 and NFκB were responsible for COX-2 transcription induced by TNFα. The 371-bp proximal region used in the present study contains an NF-IL-6, but no NFκB response element. This suggests that NFκB is not crucial for COX-2 transcription induced by IL-1 and IL-6. IL-6-induced gp130 signals activate both the tyrosine kinase JAK2-signal transducer and activator of transcription (STAT) cascade and Ras-dependent MAP kinase cascade, and the latter cascade leads to the activation of NF-IL-6 (35). Further studies are needed to identify the elements regulated by IL-1 and IL-6 in the mouse COX-2 gene promoter.

Bone loss caused by estrogen deficiency is thought to be due to the increased bone resorption stimulated by cytokines such as IL-1 and IL-6. Pacifi and his co-workers reported that the administration of IL-1 receptor antagonist to OVX rats decreased bone resorption (17). Manolagas and his co-workers reported that the increased bone resorption in OVX mice was restored by giving mice anti-IL-6 antibody in vivo (19). We reported that bone-resorbing activity present in bone marrow supernatants from OVX mice was much higher than that from sham mice (20) and was suppressed by indomethacin as well as antibodies against IL-1 and IL-6. The bone marrow supernatants from OVX mice had a greater effect than the supernatants from sham mice on inducing COX-2 expression and PG synthesis in mouse calvarial cultures (21). These results suggest that synergistic effects of IL-1 and IL-6 on PG2 synthesis may be critical in the mechanism of bone resorption in estrogen deficiency. It was also reported that bone loss in rheumatoid arthritis patients was linked to the increased levels of bone-resorbing cytokines, including IL-1, IL-6, and TNFα in synovial fluids (24, 25, 36). Expression of COX-2 mRNA occurred in synovial cells of rheumatoid arthritis patients (37), suggesting that PGs are involved in bone resorption in rheumatoid arthritis patients as well.

In conclusion, IL-6 induces COX-2 transcription in the presence of sIL-6R in mouse osteoblasts, and this induction appears to be involved in the IL-6-induced osteoclast formation. The additive stimulation of osteoclast differentiation by the submaximal doses of IL-1 and IL-6 may be explained by the greater induction of COX-2-dependent PG synthesis by a combination of these cytokines, although COX-1 may also play a role. How IL-1 and IL-6 interact to induce COX-2 transcription is interesting and is currently under investigation in our laboratories.

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